

ORIGINAL RESEARCH

Alterations of the bile microbiome in primary sclerosing cholangitis

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ABSTRACT

Background Patients with primary sclerosing cholangitis (PSC) display an altered colonic microbiome compared with healthy controls. However, little is known on the bile duct microbiome and its interplay with bile acid metabolism in PSC.

Methods Patients with PSC (n=43) and controls without sclerosing cholangitis (n=22) requiring endoscopic retrograde cholangiography were included prospectively. Leading indications in controls were sporadic choledocholithiasis and papillary adenoma. A total of 260 biospecimens were collected from the oral cavity, duodenal fluid and mucosa and ductal bile. Microbiomes of the upper alimentary tract and ductal bile were profiled by sequencing the 16S-rRNA-encoding gene (V1–V2). Bile fluid bile acid composition was measured by high-performance liquid chromatography mass spectrometry and validated in an external cohort (n=20).

Results The bile fluid harboured a diverse microbiome that was distinct from the oral cavity, the duodenal fluid and duodenal mucosa communities. The upper alimentary tract microbiome differed between PSC patients and controls. However, the strongest differences between PSC patients and controls were observed in the ductal bile fluid, including reduced biodiversity (Shannon entropy, $p=0.0127$) and increase of pathogen *Enterococcus faecalis* ($FDR=4.18 \times 10^{-5}$) in PSC.

Enterococcus abundance in ductal bile was strongly correlated with concentration of the noxious secondary bile acid tauroolithocholic acid ($r=0.60$, $p=0.0021$).

Conclusion PSC is characterised by an altered microbiome of the upper alimentary tract and bile ducts. Biliary dysbiosis is linked with increased concentrations of the proinflammatory and potentially cancerogenic agent tauroolithocholic acid.

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a cholestatic liver disease of unknown origin, which is characterised by progressive fibrotic strictures of bile ducts and ulcerative lesions of the bile duct mucosa.^{1–3} PSC is strongly associated with a unique phenotype of IBD. Patients with PSC suffer from an increased mortality, mainly due to increased risk of cholangiocarcinoma and cancers of the gallbladder and

Significance of this study**What is already known on this subject?**

- Primary sclerosing cholangitis (PSC) is associated with alterations of the colonic microbiome.
- Opposed to traditional understanding, human bile is a non-sterile environment (even in healthy humans).

What are the new findings?

- Composition of bile duct microbiome is different from other upper digestive sites such as the oral cavity and duodenum.
- PSC patients display ecological alterations of ductal bile, including reduced biodiversity and expansion of pathogenic bacteria.
- Microbial dysbiosis in PSC is associated with an increase of the proinflammatory and potentially cancerogenic bile acid tauroolithocholic acid.

How might it impact on clinical practice in the foreseeable future?

- Microbial dysbiosis of the ductal bile fluid highlights the potential pathophysiological importance of the biliary microbiome in PSC.
- This finding encourages precise modulation of biliary microbial colonisation to reduce the risk of adverse health outcomes associated with PSC.

colon.^{1,2} Liver transplantation is the only curative treatment option available.

The pathogenesis of PSC remains obscure. Genetic studies support the hypothesis that PSC is an autoimmune disorder, but male preponderance and poor response to immunosuppression render it different from typical autoimmune diseases.^{1,2} Multiple lines of evidence point at commensal bacterial communities as key players in the pathophysiology of PSC.^{2,3} Recent next-generation sequencing (NGS) studies revealed an altered gut bacterial microbiome in patients with PSC, both at the faecal and mucosal level, which was different from both healthy individuals and patients with UC.^{4–10} Until the advent of NGS, healthy human bile has been



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widely considered sterile. Nevertheless, earlier culture-based studies implicated bacteria inhabiting the bile fluid in T helper cell type 17 immune response and clinical outcomes in PSC.^{11 12} A recent NGS study showed that both patients with PSC and controls without sclerosing cholangitis harbour a diverse bile microbiome.¹³

Bile acids are the major organic solutes of human bile.¹⁴ Bile acids are believed to play a crucial role in pathogenesis of PSC, although evidence of a 'toxic' bile composition per se in PSC patients is lacking.^{15 16} Since conversion of the primary bile acids cholic acid and chenodeoxycholic acid into the secondary and potentially noxious bile acids deoxycholic acid and lithocholic acid is thought to be primarily driven by the bacterial gut microbiome, microbial dysbiosis is expected to exert a profound influence on the bile acid pool and in turn mucosal homeostasis in PSC.¹⁵ However, to the best of our knowledge, the link between bile acids and the microbiome of the bile fluid has not been investigated so far.

In the present study, we aimed to investigate the bacterial ecology of the upper alimentary tract as well as ductal bile fluid in selected cohorts of patients with PSC and controls undergoing endoscopic retrograde cholangiography (ERC). Furthermore, we assayed the entire bile fluid bile acid profiles of the respective cohorts in order to analyse potential interactions between bile fluid bacteria and bile acids in PSC.

METHODS

Patient recruitment and biospecimen acquisition

All patients with PSC and controls were recruited at the University Medical Center Hamburg-Eppendorf. The diagnosis of PSC was established based on the presence of typical biliary lesions on cholangiography, liver biopsy (if available) and exclusion of secondary causes of sclerosing cholangitis, according to most recent guidelines.^{17 18}

Exclusion criteria were acute bacterial cholangitis on index ERC, previous ERC within the last 12 months, patient age <18 years, severe medical comorbidity, small duct PSC and any evidence of secondary sclerosing cholangitis. Patients were required not having received any antibiotic treatment during 6 months before ERC as this time interval is expected to be sufficient for broad microbiome recovery after antibiotic treatment.^{19 20} In order to reduce the influence of geography and diet, all recruited participants were residents of Northern Germany for years and consumed a mixed Western style diet.

In total, 65 patients were eligible for the analysis (PSC: n=43, controls: n=22). A detailed patient description is provided in table 1. Indications for ERC in patients with PSC included cholestasis or suspicion of dominant strictures on MRI. In controls, PSC was excluded by cholangiography and clinical follow-up. Detailed indications for ERC for both cohorts and diagnoses of the control cohort can be found in online supplementary table 1. Intervention and specimen acquisition are described in the online supplementary methods in detail. During ERC, bile fluid aspiration was performed before application of contrast media or peri-interventional antibiotic prophylaxis, which is standard in our unit. There were no patients with biopsy proven bile duct dysplasia or cholangiocarcinoma.

All participants provided written informed consent. The study was conducted in compliance with the 1975 Declaration of Helsinki.

Table 1 Clinical patient characteristics

	PSC	Controls	P value
Patients, n	43	22	NA
Female, n (%)	16 (37)	11 (50)	>0.1
Age, years	39 (20–55)	55 (22–89)	<0.01
BMI, kg/m ²	23.38 (15.9–35.4)	25 (18.7–41.3)	>0.1
Liver cirrhosis,* n (%)	7 (16.3)	1 (5)	>0.1
Previous ERC, n (%)	29 (67.4)	1 (5)	<0.001
Previous bacterial cholangitis, n (%)	2 (5)	0	>0.1
Transient elastography, kPa	8.7 (3.5–66.4)	NA	NA
Disease duration, years	8 (0–28)	†	NA
IBD, n (%)	29 (67.4)	0	<0.001
Bilirubin, mg/dL	1 (0.2–5.8)	0.8 (0.2–7.0)	>0.1
ALT, U/L	76.5 (9–274)	49.0 (15–580)	>0.1
ALP, U/L	244 (49–961)	129 (53–539)	<0.05
CRP, g/dL	<5 (<5–39)	<5 (<5–61)	>0.1
WCC, 10 ³ /μL	6.1 (3.3–17.2)	7.9 (3–16.6)	<0.01
UDCA, ‡ n	40 (93)	2 (9)	<0.001
Azathioprine, n (%)	8 (18.6)	0	<0.05
Mesalazine, n (%)	16 (37.2)	0	<0.001
Corticosteroids, n (%)	3 (7)	2 (9)	>0.1
Proton pump inhibitors, n (%)	2 (5)	2 (9)	>0.1

All data are provided for the time of index ERC. Median and range or counts and percentages are reported, respectively. Continuous variables were tested by Wilcoxon rank-sum test. Nominal variables were tested either with χ^2 test or Fisher's exact test.

*Liver cirrhosis was diagnosed based on criteria of clinical signs, imaging, transient elastography and biopsy (if available).

†All control subjects received first diagnosis of biliary obstruction.

‡All patients with PSC treated with UDCA received a daily dose of 15–20 mg/kg.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; BMI, body mass index; CRP, C reactive protein; ERC, endoscopic retrograde cholangiography; NA, not available; UDCA, ursodeoxycholic acid; WCC, white cell count.

Sequencing, bioinformatics and bile acid assay

A detailed description is provided in the online supplementary methods. In brief, DNA extraction and sequencing of the variable regions V1–V2 of the 16S rRNA gene on Illumina MiSeq (Illumina Inc, San Diego, California, USA) were performed as described previously.²¹ DADA2 was used for meta-taxonomic bioinformatics, a method that retrieves unique ribosomal sequence variants. Sequences abundance was normalised according to the GMPR method.²² SILVA was chosen as the taxonomic reference database (version 132; <https://www.arb-silva.de>). Tax4Fun was employed for inferred metagenome profiling against canonical pathways of the Kyoto Encyclopaedia of Genes and Genomes (<http://tax4fun.gobics.de>).

High-performance liquid chromatography mass spectrometry was performed, essentially as described previously (online supplementary methods).²³

Data analysis

All analyses were carried out with R (version 3.4.3, R Foundation for Statistical Computing, Vienna, Austria). A detailed account is given in the online supplementary methods section. To summarise, standard community ecology analyses were carried out mainly using vegan. Single bacteria differential abundance testing was conducted with negative-binomial generalised linear models from the MASS package and negative-binomial hurdle models, which account for zero inflation in count data, implemented in the pscl package. To exclude that contamination via the endoscopic route accounted for the abundance patterns observed, normalised distributions of each taxon at the respective proximal sampling sites were included as covariables in the respective models, alongside clinical variables with significant differences between cohorts

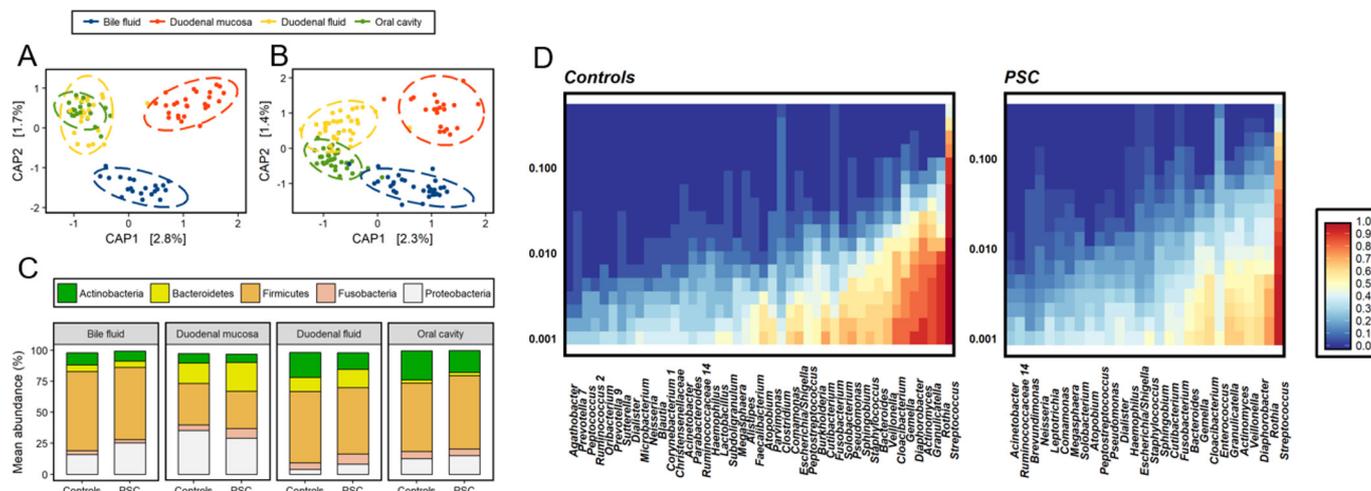


Figure 1 β -diversity between oral (n=65), duodenal fluid (n=65), duodenal mucosa (n=65) and bile fluid (n=65) in (A) controls and (B) patients with PSC, measured by constrained analysis of principal coordinates on Bray-Curtis distance. (C) Bar plots representing differential phylum relative abundances in the respective body sites. The phylum Proteobacteria was statistically significantly increased in bile fluid samples of patients with PSC compared to controls (Welch's t-test, $p=0.0384$). (D) Core microbiota analysis of bile fluid communities on the genus level. The heatmaps display the percentage of patients (colour gradient; 1=100%; n=65 patients in total) positive for the respective genus. The x-axis lists taxon names that are found at a given relative abundance (y-axis; detection threshold; 0.1=10%). Patients with PSC displayed a less diverse core composition and decline of many core taxa found in control patients.

(table 1), where possible. An α -level of <0.05 was set as the threshold for statistical significance. P values were adjusted for the false discovery rate (FDR), where necessary.

RESULTS

Ductal bile fluid harbours a unique and diverse microbiome

Since it was unknown whether the microbial ecology of any of the upper alimentary sites investigated resembles the bile fluid microbiome, we first examined differences of the microbiome structure between the oral cavity, duodenal fluid, duodenal mucosa and bile fluid by constrained analysis of principal coordinates using Bray-Curtis distance (figure 1A,B). Microbial communities obtained from duodenal mucosal biopsies clustered separately from the other sites (ANOVA-like permutation test, $p<0.01$, respectively). While in both cohorts oral and duodenal fluid communities were similar ($p>0.1$), there was a clear separation between the bile fluid microbiome and all other communities ($p\leq 0.01$, respectively).

We next explored the community structures on the phylum level (highest taxonomic hierarchy level). Only five phyla accounted for virtually the entire microbiome in all sites, which are Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria. Comparing patients with PSC and controls, we observed no significant differences in phyla abundances in the oral cavity and duodenal sites (Welch's t-test, all $p>0.05$). Regarding the ductal bile fluid, Proteobacteria showed a significant increase in patients with PSC compared to controls (mean relative abundance=25% vs 12%; $p=0.0384$; figure 1C). Other biliary phyla in patients with PSC showed an abundance similar to controls ($p>0.1$, respectively).

Since the baseline structure of the healthy bile fluid core microbiome is unclear, we investigated the biliary core microbial profiles of the control cohort and patients with PSC on the genus taxonomic level.²⁴ Both groups harboured a diverse core microbiome with *Streptococcus* as the predominant genus (figure 1D). However, patients with PSC displayed decreased richness of the core microbiome evidenced by decreased prevalence of genera with $>0.1\%$ relative abundance. These results highlight the

deviation in PSC from a stable core bile microbial composition in a healthy state (figure 1D).

Altered upper alimentary and bile duct fluid microbiome in PSC

We compared the within-sample diversity (α -diversity) between patients with PSC and controls in the different body sites. The bile fluid microbial communities of patients with PSC displayed a reduced average α -diversity compared with controls (Shannon entropy; Welch's t-test, $p=0.0127$; figure 2A). Significant differences of α -diversity were not observed in the other body sites, which indicates that the biliary microbiome in PSC is more severely altered than the other upper digestive communities tested. We observed significant differences in the overall community structure (β -diversity) between patients with PSC and controls in the oral cavity, duodenal fluid and bile duct fluid (ANOVA-like permutation test, $p\leq 0.01$, respectively; figure 2B) and a trend towards difference in the duodenal mucosal communities ($p=0.064$).

Expansion of pathogens in bile fluid of patients with PSC

We tried to identify differentially abundant bacteria on the lower taxonomic hierarchy levels (genus and species) between patients with PSC and controls.

We first investigated differential abundance patterns in the oral cavity, duodenal fluid and duodenal mucosa (online supplementary results and online supplementary figure 1). Notably, we found a pronounced over-representation of the species *Escherichia coli* ($\log_2FC=4.19$, $FDR=6.02\times 10^{-29}$) and *Veillonella dispar* ($\log_2FC=1.7$, $FDR=0.0034$) in duodenal mucosal biopsies of patients with PSC.

The biliary communities of patients with PSC showed the most extensive differences on the single taxa level compared with controls (figure 2C). The strongest increase was observed for *Enterococcus* ($\log_2FC=8.13$, $FDR=4.18\times 10^{-5}$), followed by *Staphylococcus* ($\log_2FC=6.71$, $FDR=4.07\times 10^{-6}$). Among other bacteria, *Neisseria* ($\log_2FC=5.97$, $FDR=7.63\times 10^{-5}$) was also overabundant in patients with PSC.

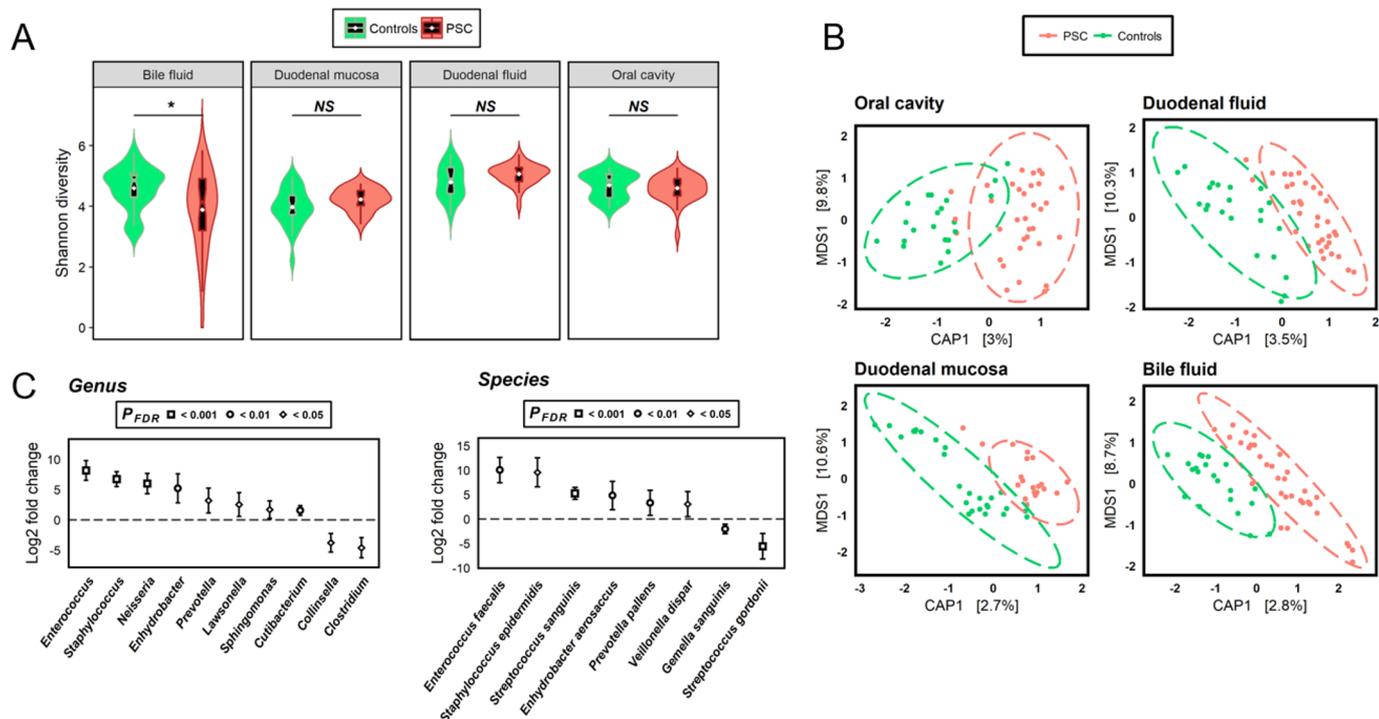


Figure 2 (A) Violin plots for Shannon α -diversity index stratified by diagnosis and body site. Only the bile fluid communities displayed a significantly reduced α -diversity in PSC (Welch's t-test, $p=0.0127$); *, $p<0.05$. (B) Differences of microbial β -diversity between patients with PSC and controls in the different body compartments. β -diversity was visualised by constrained analysis of principal coordinates using Bray-Curtis distance. (C) Single taxa that were determined as differentially abundant between patients with PSC and controls in bile fluid. Log₂ fold changes are displayed for genus and species level differential abundance. Statistical significance was determined by negative binomial generalised linear models or hurdle models. Positive log₂ fold changes indicate increased abundance in PSC. NS, not significant; P_{FDR} , p value corrected for the false discovery rate; PSC, primary sclerosing cholangitis.

Regarding the differential abundance of bile fluid species (figure 2C), *Enterococcus faecalis* showed the strongest increase in patients with PSC ($\log_2FC=10.01$, $FDR=0.0054$), followed by *Veillonella dispar* ($\log_2FC=3.03$, $FDR=0.0256$).

Bile samples obtained within our study were sent for standard culture. We compared the results obtained by NGS with bile culture results in patients with PSC and controls. There was no significant difference in the rate of positive bacterial bile cultures (46% vs 47% with positive culture; χ^2 test, $p>0.1$). Patients with PSC showed a trend towards an increased rate of culturally detected known pathogenic bacteria in bile fluid (*Enterococcus spp.*, *Klebsiella spp.*, *Enterobacter cloacae*, *Citrobacter freundii* or *Staphylococcus spp.*; 23% vs 5%; Fisher's test, $p=0.082$).

Previous ERC has minor influence on bile duct bacterial composition in PSC

We aimed to identify clinical factors that may influence bile fluid bacterial communities in patients with PSC.

We first investigated potential associations with overall ecologic indices in a multivariate approach, including the variables sex, body mass index (BMI), presence of PSC-associated colitis, bilirubin, transaminases, alkaline phosphatase (ALP) and leukocytes levels, liver stiffness measured by transient elastography (TE), previous bacterial cholangitis and previous ERC.

These variables were neither significantly associated with α -diversity using best subset selection by leaps algorithm ($p>0.05$, respectively), nor were they significant predictors of β -diversity using stepwise forward selection for constrained ordination ($p>0.05$, respectively; online supplementary methods).

Most single bacteria identified over-represented in patients with PSC in the previous section did not show any significant differential abundance between PSC patients with or without previous ERC ($FDR>0.05$, respectively). However, among bacteria over-represented in PSC, *Staphylococcus* ($\log_2FC=7.47$, $p=0.004$) and *Streptococcus sanguinis* ($\log_2FC=9.15$, $p=5.33\times 10^{-4}$) were over-represented in patients with PSC who formerly received ERC.

Altered metabolic functional profiles of bile fluid microbiome in PSC

Since metagenomic shotgun sequencing was hindered by the relatively large amount of human sequences in the bile fluid, we inferred the functional profiles from the 16S profiles (online supplementary methods).

Of the 321 pathways recovered in the bile duct fluid, 52 showed an altered expression (16.2%, 34 underexpressed, 18 overexpressed; figure 3A). Corresponding to the reduced α -diversity, we observed an extensive loss of basic microbiome functions such as 'tryptophan metabolism' ($FDR=1.72\times 10^{-4}$) or 'biosynthesis of amino acids' ($FDR=2.24\times 10^{-3}$). We observed an increase in several potentially pathogenic bacterial pathways, including 'shigellosis' ($FDR=5.12\times 10^{-5}$), '*Salmonella* infection' ($FDR=3.01\times 10^{-3}$) and 'pathogenic *E. coli* infection' ($FDR=5.11\times 10^{-3}$).

Metabolic pathways were differently distributed in patients with PSC and controls, and also in the other upper digestive tract sites, for example, an increase of 'biofilm formation by *Escherichia coli*' in the oral cavity ($FDR=0.0190$) and the duodenal

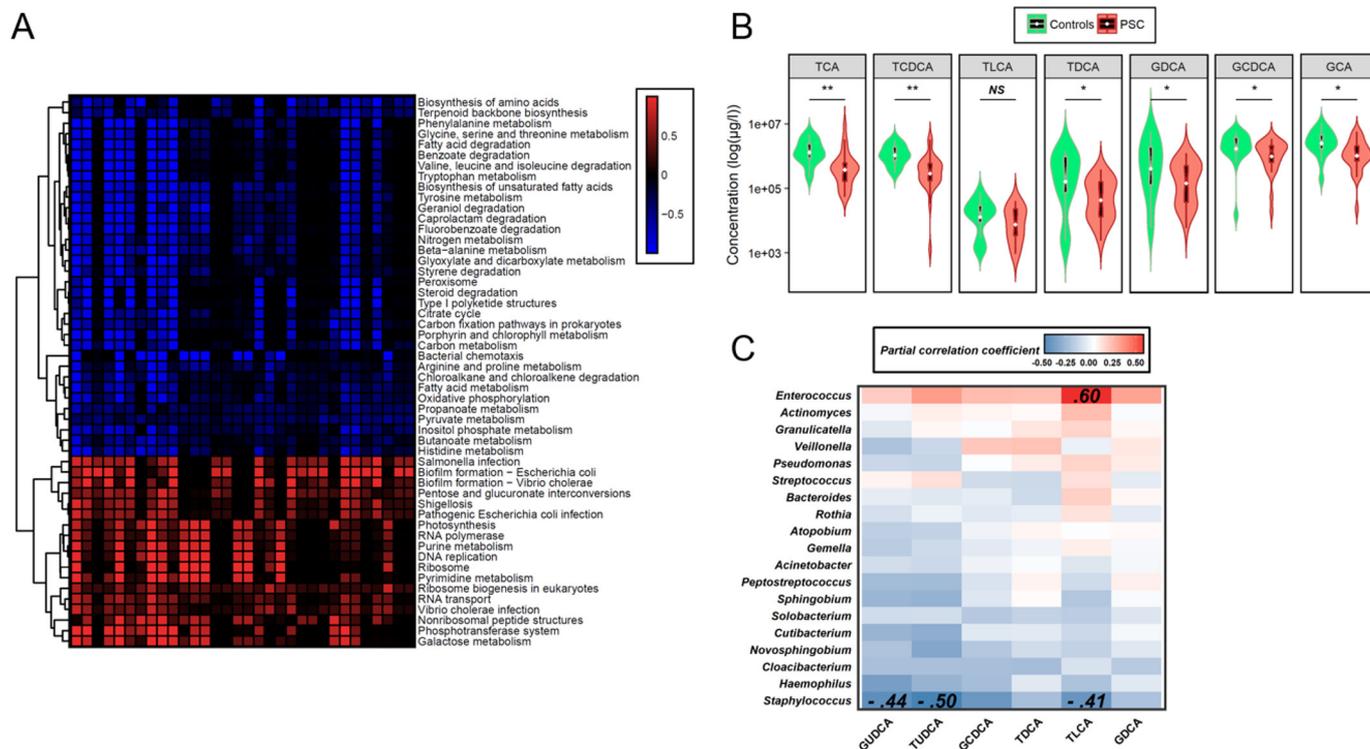


Figure 3 (A) Heatmap showing differentially abundant microbial metabolic pathways in the bile fluid. Only pathways with significant difference at $FDR < 0.05$ are shown. Each column represents an individual patient with PSC. The colour code represents the gage test statistic and direction of statistically significant differential abundance (red indicates over-representation, blue signals under-representation). (B) Bile acid concentrations in the bile fluid compared between patients with PSC and controls. The concentrations of most bile acids were reduced in patients with PSC, except for TLCA. (C) Heatmap showing partial correlation coefficients in patients with PSC between bacterial genera of the bile fluid and bile acids, which were selected using canonical correlation with penalised matrix decomposition. Partial correlations were calculated adjusting for sex, body mass index, alkaline phosphatase levels and transient elastography measures (a proxy for liver fibrosis). The coefficients are additionally displayed in numbers only for statistically significant correlations. We observed a strong and statistically significant correlation between *Enterococcus* and TLCA ($p = 0.0021$). GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; NS, not significant; PSC, primary sclerosing cholangitis; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, tauroolithocholic acid; *, $p < 0.05$; **, $p < 0.01$.

fluid ($FDR = 1.17 \times 10^{-10}$), as well as an increase of ‘lipopolysaccharide biosynthesis’ ($FDR = 1.89 \times 10^{-4}$) and ‘bacterial invasion of epithelial cells’ ($FDR = 0.0087$) in the duodenal fluid.

Altered bile acid concentrations and noxious lithocholic acid levels associated with bile dysbiosis in PSC

We aimed to investigate if microbiome composition was associated with bile acid composition in the bile fluid.

Relative bile acid concentrations in controls were as expected from the literature with little difference in patients with PSC (online supplementary figure 2). As expected from the treatment of patients with PSC, the absolute concentrations of ursodeoxycholic acid (UDCA) conjugates were greatly increased in PSC patients (measured in $\mu\text{g/L}$; Welch’s t-test, $p < 0.0001$, respectively). Most other bile acids showed reduced absolute concentrations in PSC samples ($p < 0.05$, respectively). However, tauroolithocholic acid (TLCA), a potentially noxious agent, was the only bile acid with similar concentrations between patients with PSC and controls ($p > 0.05$, figure 3B).

To exclude that the reduced absolute bile acid concentrations in patients with PSC are explained by UDCA treatment, we analysed the biliary bile acid profiles in an independent cohort of 20 patients with PSC without UDCA treatment from the Norwegian PSC Research Center, Oslo. Here, we observed the same trend towards reduced single bile acid concentrations (online

supplementary figure 3), suggesting that this observation relates, at least partly, to PSC pathophysiology itself.

We tried to establish relationships between the bile fluid microbiome and bile acid concentrations in the PSC cohort. To reduce the burden of multiple testing, we first identified variables that maximised the correlation between bile fluid microbial genera abundances and bile acid concentrations by sparse canonical correlation analysis (PMA package, online supplementary methods). Next, we tested partial correlations between genera and bile acids adjusting for sex, BMI, ALP and TE levels (figure 3C). The strongest correlation was observed between *Enterococcus* and TLCA ($r = 0.60$, test for zero partial association, $p = 0.0021$).

Thus, the biliary genus with the strongest increase in PSC, *Enterococcus*, was associated with an increase in the noxious and potentially carcinogenic bile acid TLCA.

DISCUSSION

PSC is a disease that mainly affects the bile ducts, which represent a large mucosal barrier within the body.¹ An altered microbiome may significantly contribute to the non-genetic risk associated with PSC.^{2 15} In the present study, we detected differences in the microbial composition between patients with PSC and controls without sclerosing cholangitis in the oral cavity, the duodenum and the ductal bile fluid. Adding to previously reported changes

of the faecal and colonic mucosal microbiome in PSC, our study shows that the upper alimentary tract and the bile ducts of PSC patients are likewise affected by microbial dysbiosis. The biliary microbiome in patients with PSC exhibited the most extensive alterations that were evident on both the taxonomic and inferred functional levels. *Enterococcus*, the genus with the strongest increase in bile ducts of patients with PSC, was associated with lithocholic acid, a noxious and potentially carcinogenic bile acid.

In the single previous study on the biliary microbiome in PSC, the authors detected only slight microbial alterations in patients with either biliary dysplasia or cholangiocarcinoma, while patients without disease complications showed virtually no microbial differences compared with controls.¹³ In contrast, we observed significant biliary microbial alterations with considerable effect sizes in well-characterised PSC patients without dysplasia or carcinoma. The significant results may have been facilitated by a more refined statistical approach and a well-controlled design with an extended period after last antibiotic or ERC treatment. Furthermore, additionally sequencing the microbiome from proximal upper digestive sites allowed us to control for the effect of bile fluid contamination via the endoscopic route, which may have been a shortcoming of the previous study.¹³ Clearly, the biliary microbiome was found to be distinct from the duodenal mucosal or luminal microbiome, demonstrating that duodenal fluid cannot be used as a proxy in studies aiming to address the biliary microbiome.

We observed a significant increase of the facultative anaerobic phylum Proteobacteria in the bile fluid of patients with PSC. The abnormal bloom of Proteobacteria, which comprises many known human pathogens, such as members of the Enterobacteriaceae family, typically occurs in association with increased epithelial oxygen availability and is therefore believed to be a hallmark of inflammation, epithelial dysfunction and disease.²⁵ Furthermore, we observed a reduced average biodiversity of the bile fluid microbiome in patients with PSC. From an ecological standpoint, decreased biodiversity is a critical state that leads to a loss of ecosystem resilience and favourable ecosystem functions.²⁶

Regarding the results of bile fluid cultures, known pathogens of cholangitis were detected more frequently in samples of patients with PSC. This trend was confirmed on the 16S rRNA gene level, where we observed an over-representation of potential pathobionts such as *Enterococcus spp.*, *Prevotella spp.*, *Staphylococcus spp.*, *Lawsonella spp.*²⁷ and *Cutibacterium*. Here, *E. faecalis* showed the most marked increase in patients with PSC. *Enterococcus* has previously been shown to be more abundant in faeces of patients with PSC.⁶ *E. faecalis* has been associated with epithelial barrier damage and mucosal inflammation due to its production of matrix metalloproteinases.²⁸ In addition, biliary isolates of *E. faecalis* have been shown to induce T helper type 17 immune responses in peripheral blood of patients with PSC.¹² In a recent report, *E. gallinarum* was among the gut pathobionts translocating into mesenteric lymph nodes and driving T helper 17 cells mediated hepatobiliary injury in a model of PSC.²⁹

We detected an increased abundance of *Veillonella dispar* on the duodenal mucosa and in the bile duct fluid of patients with PSC. This pathogen has previously been repeatedly detected as over-represented in faecal communities of patients with PSC.^{9–10} In previous studies on Crohn's disease, a disease that shares considerable overlap with PSC in clinical phenotype, *Veillonella spp.* alongside *Enterococcus spp.* was associated with an increased risk of recurrent disease after surgical resection and predisposition to penetrating complications in paediatric patients.^{30–31} Thus, we believe that the potential functional and

prognostic role of *Veillonella* and *Enterococcus* in PSC should be studied in the intestine and within the bile ducts in future follow-up studies.

Interestingly, we found a marked increase of *E. coli* on the duodenal mucosal surfaces of patients with PSC. An increased prevalence of mucosa-adherent *E. coli* is well recognised in IBD, where it is believed to instigate mucosal injury.³² Furthermore, *Escherichia* has been shown to produce cysteamine, which is the most potent inducer of vascular adhesion protein (VAP)-1. Elevated levels of soluble VAP-1 have been linked to poor prognosis in patients with PSC, therefore, providing a link between overgrowth of *E. coli* and clinical outcomes in PSC.³³

The expansion of bacterial pathogens was reflected on the level of the upper alimentary tract and biliary microbiome functional profiles, in which an increase of invasive and proinflammatory bacterial metabolic capacity was observed. Among increased microbial pathways, we found epithelial cell adhesion and invasion as well as synthesis of lipopolysaccharides, molecules that may drive biliary epithelial inflammation in PSC.³⁴ Since the bile duct mucosa is the primary site of inflammation in PSC, these results point towards a possible contribution of an altered biliary microbiome to cholangiocyte and bile duct mucosal damage. Furthermore, the extensive loss of function in the PSC bile fluid communities might reflect a decline of beneficial microbial contribution to bile duct mucosal homeostasis. This result may relate to the recent discovery of an altered bile metabolome in PSC.³⁵

Examining the bile acid profiles, we found a reduced bile acid pool in patients with PSC, except for the secondary bile acid TLCA. Our observation is in accordance with previously reported globally reduced bile acid concentrations in patients with obstructive cholestasis due to PSC.¹⁶ The causes of this alteration are unknown, but reabsorption of bile acids, reduced synthesis and dilution of stagnant bile are obvious explanations¹⁶ and may represent protective mechanisms in cholestatic liver disease. These results were validated in a geographically separated PSC cohort without UDCA treatment. Lithocholic acid is a rare example of a noxious endobiotic which, together with its conjugates, is considered the most harmful bile acid.³⁶ Lithocholic acid causes segmental bile duct obstruction, destructive cholangitis and periductal fibrosis³⁷ and exerts cancerogenic effects.³⁸ For PSC, it was proposed that bile duct injury induced by bile acids is caused by more vulnerable bile ducts rather than by absolute excess of noxious bile components.^{3–16} Therefore, physiological TLCA concentrations may already cause bile duct injury in PSC patients with cholestasis and a damaged mucosal barrier. Interestingly, TLCA levels were strongly correlated with *Enterococcus* abundance in PSC samples. *E. faecalis* expresses higher bile salt hydrolase activity than other human commensal microbes.³⁹ Bile salt hydrolases catalyse the crucial step of deconjugation in the process of converting primary to secondary bile acids. Hence, the dysbiotic excess of *Enterococcus spp.* may be causally linked to secondary bile acid levels with both proinflammatory and cancerogenic impact on patients with PSC. Previously, the increased malignancy rate and disease progression in patients with PSC imposed by high-dose UDCA treatment has been linked to increased lithocholic acid levels resulting from conversion of UDCA.⁴⁰ As both the abundance of *Enterococcus* and concentration of TLCA were heterogenous among patients with PSC, it is an interesting question to be addressed in follow-up studies whether patients with increased TLCA levels represent a subgroup at risk for adverse outcomes such as cholangiocarcinoma.

Our study has strengths and limitations. It is difficult to study human ductal bile and almost impossible to obtain samples either in a sterile way or without prior perioperative antibiotic prophylaxis. By controlling for the microbiome in the oral cavity and duodenum, we tried to circumvent this technical problem. Selecting proper controls is challenging, since ERC is a method associated with health risks for the examined individual and thus cannot be performed on healthy volunteers for ethical reasons. Although we required no antibiotic treatment during 6 months before biliary sampling during ERC, an effect of prior antibiotic treatment on the biliary microbiome cannot be excluded entirely. As most patients with PSC were treated with UDCA, an impact on the biliary microbiome cannot be ruled out. Nevertheless, in previous reports, there was no evidence for an impact of UDCA on the faecal microbiota in patients with PSC.^{6,9} In addition, species level and functional information obtained through 16S rRNA gene sequencing are not as reliable as by employing shotgun metagenomic sequencing of the entire DNA. However, due to the low abundance of bacterial DNA as compared with human DNA in bile fluid, metagenomic shotgun sequencing could not be performed successfully on our samples at reasonable sequencing depths. Our microbiome analysis is confined to an ethnically, geographically and dietary homogenous single-centre cohort. Future studies should assess the robustness of the alterations observed by including international multicentric cohorts and by analysing potential impact of differing diets. While a comparison of the biliary microbiome between patients with PSC and other chronic liver diseases may add to the understanding of disease-specific pathophysiological implications of microbial alterations, obtaining bile via ERC from patients with parenchymal liver diseases is not feasible for ethical reasons. Therefore, caution is warranted regarding the specificity of our result for PSC. Many microorganisms cannot be grown using routine cultivation methods. The present results show that an NGS approach surpasses the constraints of standard bile culture, as has been previously demonstrated for numerous human and environmental habitats.⁴¹

In summary, the present study demonstrates a dysbiosis in the microbial communities of the upper alimentary tract and bile ducts of patients with PSC, with the most significant alterations found in the bile fluid. We hypothesise that changes in the biliary microbiome may contribute to PSC pathogenesis by enhancing the damage of bile duct mucosa and potentially by effects on the concentration of the noxious bile acid lithocholic acid. As our study is cross-sectional and therefore cannot prove causality, this hypothesis should be further investigated in future follow-up and more functional experimental studies. Multiple lines of evidence point towards microbial factors influencing clinical outcomes in PSC.^{11,33,42–44} As advances in microbiome research are spurring the development of precision medicine interventions, such as techniques of finely tuned control of bacterial strain abundance⁴⁵ and selective inhibition of pathogen expansion in inflammation,⁴⁶ our results may provide a starting-point for clinical studies on the bile microbiome in PSC.

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